In vivo switching of human melanoma cells between proliferative and invasive states

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Abstract: Metastatic melanoma represents a complex and heterogeneous disease for which there are no therapies to improve patient survival. Recent expression profiling of melanoma cell lines identified two transcription signatures, respectively, corresponding with proliferative and invasive cellular phenotypes. A model derived from these findings predicts that in vivo melanoma cells may switch between these states. Here, DNA microarray-characterized cell lines were subjected to in vitro characterization before s.c. injection into immunocompromised mice. Tumor growth rates were measured and postexcision samples were assessed by immunohistochemistry to identify invasive and proliferative signature cells. In vitro tests showed that proliferative signature melanoma cells are faster growing but less motile than invasive signature cells. In vivo proliferative signature cells initiated tumor growth in 14 +/- 3 days postinjection. By comparison, invasive signature cells required a significantly longer ($P < 0.001$) period of 59 +/- 11 days. Immunohistochemistry showed that regardless of the seed cell signature, tumors showed evidence for both proliferative and invasive cell types. Furthermore, proliferative signature cell types were detected most frequently in the peripheral margin of growing tumors. These data indicate that melanoma cells undergo transcriptional signature switching in vivo likely regulated by local microenvironmental conditions. Our findings challenge previous models of melanoma progression that evoke one-way changes in gene expression. We present a new model for melanoma progression that accounts for transcription signature plasticity and provides a more rational context for explaining observed melanoma biology.

DOI: https://doi.org/10.1158/0008-5472.CAN-07-2491

TITLE

In vivo switching of human melanoma cells between proliferative and invasive states.

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ABSTRACT

Transcription profiling of melanoma cell lines previously identified two transcription signatures predicted to respectively correspond with proliferation and invasion. Here we identify Microphthalmia-associated transcription factor (Mitf) as a useful marker for signature membership and demonstrate that knockdown of Mitf alters signature phenotype. Proliferative and invasive signature cell lines were used in xenograft experiments and resulting tumors were assessed for Mitf expression by immunohistochemistry. All tumors, irrespective of the signature type injected, displayed immunohistochemical evidence for both proliferative and invasive signature melanoma cells. These data indicate that melanoma cells undergo bi-directional transcriptional signature switching *in vivo* which is likely regulated by local microenvironmental conditions. The findings challenge previous models of melanoma progression which evoke one-way changes in gene expression.

SIGNIFICANCE

Current molecular models for melanoma link linear gene expression change with clinical progression, a paradigm which has informed nearly all molecular studies of the disease. We build on previous experiments, where we showed that melanoma cells may present with either of two distinct transcription signatures respectively related to proliferative and invasive aspects of the disease, to demonstrate that *in vivo* these signatures are interchangeable rather than fixed states. We construct a new model which describes melanoma progression as a result of melanoma cells oscillating between transcriptional programs of proliferation and invasion.

INTRODUCTION

Metastatic stage melanoma is an aggressive disease that few patients survive for more than two years. Compounding this, scores of clinical trials testing different adjuvant therapies have brought no significant improvement in the survival outlook for these patients (Sasse et al., 2007). One possible explanation for this is that melanoma is a heterogeneous collection of different cells, with differences between them sufficient that some cells are missed by targeted therapies. The variety of phenotypic and behavioral features melanomas present range from differing organ specificities during metastasis to changes in characteristics of motility and invasion (Fidler and Kripke, 1977). Furthermore, examination of melanoma tissues reveal various morphologies, from assorted macroscopic lesional structures to an array of microscopic cellular forms, which often complicate assessments of diagnosis and prognosis (Levene, 1980). Additionally, immunohistochemical staining regularly yields equally heterogeneous results. While most melanoma lesions will stain for a number of melanocytic markers, this is not necessarily true for all the melanoma cells within a given lesion (Banerjee and Harris, 2000). Finally, DNA microarray examination of different lesions and melanoma cell line collections reveal among them consistent taxonomies of genomic aberrations and transcriptional signatures (Curtin et al., 2005; Haqq et al., 2005; Hoek et al., 2006). The source of heterogeneity is thought to rest in the combination of how melanoma cells respond to different microenvironments and the reciprocal influence of their own molecular states. This was an idea first conceptualized to a significant degree in the "seed and soil" model put forward by Stephen Paget after his observation that particular cancer cells demonstrated tumorigenic preference for certain tissues over others (Paget, 1889; Ribatti et al., 2006). By comparison, our current molecular models for melanoma progression are comparatively homogenous. Generally accepted hypotheses concerning molecular contributions towards melanoma pathobiology assume the reasonable premise that progression of the disease is driven by a steady evolution of molecular changes within the cell (Miller and Mihm, 2006). It

is in this context that concepts of gene modification or gene expression change are invoked to provide the framework upon which molecular studies into melanoma are carried out.

Of particular recent interest has been the activity of the microphthalmia-associated transcription factor Mitf in regulating melanoma cell proliferation. In normal melanocytes Mitf is critical for melanocytic differentiation, expression of melanogenic enzymes and upregulating cyclin-dependent kinase inhibitors to drive cell cycle exit (Carreira et al., 2005; Loercher et al., 2005; Steingrimsson et al., 2004). However, in melanoma Mitf is required for proliferation and has been identified as a "lineage survival" factor prone to amplification (Carreira et al., 2006; Du et al., 2004; Garraway et al., 2005). While the contrast in the activities of Mitf in normal and transformed cells remains unexplained, there is little doubt concerning its central role in melanoma biology.

We recently explored heterogeneity of gene expression in melanoma cells, following earlier work performed by Bittner and coworkers who suggested that there may be specific transcriptional signatures delineating melanoma cell subgroups (Bittner et al., 2000). We characterized two different transcription signatures for melanoma cell lines which, based on known functions of the genes involved, defined their respective contributions to metastatic potential as either proliferative or invasive (Hoek et al., 2006). We further hypothesized that the transcription signatures represent distinct yet interchangeable states regulated by changes in signaling from the microenvironment. Critically, Mitf expression is a central feature of the proliferative signature and is absent from the invasive form. Others' *in vitro* work concerning *MITF* gene regulation have corroborated the hypothesis that its expression is important for differentiating between proliferative and invasive states (Carreira et al., 2006). To test the validity of the proliferative signature we examined Mitf's role in the proliferative signature phenotype and compared the *in vivo* tumorigenicity of these cells against those with an

invasive signature. At the same time we used immunohistochemistry to monitor Mitf and the Ki67 antigen in the resulting tumors to provide evidence of *in vivo* switching between signatures.

RESULTS

Proliferative And Invasive Transcription Signatures Yield Distinct *In Vitro* Phenotypes

To study the *in vivo* tumorigenic behaviour of melanoma cell lines with different transcriptional signatures, we selected pairs of proliferative and invasive signature melanoma cell lines based on previous genome-wide transcription profiling experiments (Hoek et al., 2006). We performed supervised hierarchical clustering of the samples using normalized signal intensity data of 105 genes tightly linked to signature (Figure 1A). Earlier experiments in which monolayers of cells were observed for surface motility had shown that proliferative signature lines were significantly less motile than invasive signature lines (Hoek et al., 2006). Also, the different signature cell types were subjected to TGFβ challenge and this showed that proliferative signature cells were significantly more susceptible to TGFβ-mediated growth inhibition than invasive signature cells (Hoek et al., 2006). We performed additional *in vitro* motility and proliferation experiments to expand this range of *in vitro* characterizations. Cell growth experiments showed a significant ($p < 0.001$) difference in proliferation rates between proliferative and invasive signature cell lines (Figure 1B). On the other hand, invasive signature cell lines plated at subconfluent densities on 8μm microporous transwell filters migrated in significantly ($p < 0.001$) higher numbers towards the lower chamber over 16 hours than identically plated proliferative cell lines (Figure 1C). With these experiments we therefore concluded that signature assignments given to cell lines according to their gene expression signature correlate appropriately with *in vitro* data in the context of our model. This model implies that the signatures differ through activation of gene expression patterns by different signal pathways and predicts that *in vivo* conditions may influence signaling to effect signature switching.

Immunohistochemistry Identifies A Signature-Specific Marker

Given that it was our intention to use immunohistochemistry to follow cell signatures *in vivo*, we selected markers according to their signature specificity. Previous analysis indicated that Mitf mRNA and protein levels are high in proliferative signature lines and at low or undetectable levels in invasive signature samples (Hoek et al., 2006). We confirmed this by performing immunohistochemistry on paraffin-embedded cultures of proliferative and invasive signature melanoma lines. Immunohistochemical staining of the different signature cell line pellets with anti-Mitf antibodies showed that in proliferative signature cell lines 93% of cells were positive for nuclear staining for Mitf while the invasive signature cell lines showed 0% positivity (Figure 2). We have not found immunohistochemical markers which unequivocally identify invasive melanoma cells. This is principally because these cells have downregulated all of the genes responsible for the melanocytic phenotype observable in proliferative signature melanoma cells. Instead, the differential in proliferation rates for the different signatures indicated that a general proliferation marker may be useful for immunohistochemical identification of signature type *in vivo*. While examination of previously published gene expression data shows that between transcriptional signature types there is no significant differential in the expression of mRNA encoding the proliferation marker Ki67 antigen, the significant difference in *in vitro* proliferation rates suggest that Ki67 antigen is likely to show a difference at the protein level. Accordingly, staining for Ki67 antigen showed that 94% of proliferative signature cells and 45% of invasive signature cells had positively stained nuclei (Figure 2). These results indicate that Mitf is a good marker for specific identification of proliferative signature cells and that Ki67 antigen is a suitable marker for identifying regions undergoing differential rates of proliferation.

Mitf Expression Reflects Signature Phenotype

In order to confirm that Mitf expression is functionally linked to signature phenotype we used siRNA to knockdown Mitf protein levels and assessed the effects *in vitro*. One *in vitro* characteristic which distinguishes between proliferative and invasive signature melanoma cells is a differential in susceptibility to TGFβ-mediated inhibition of proliferation, with proliferative signature cells being more sensitive to TGFβ than invasive signature cells (Hoek et al., 2006). Because proliferative signature cells express Mitf and invasive signature cells do not, we hypothesized that Mitf expression mediated the growth inhibitory effect of TGFβ on proliferative signature cells. We performed anti-Mitf siRNA knockdown experiments in a proliferative signature melanoma line and confirmed knockdown by Western blot analyses (Figure 3A). We found that Mitf-depletion from proliferative signature melanoma cells made them less susceptible to TGFβ-mediated growth inhibition (Figure 3B), showing that Mitf mediates the growth inhibitory effect of TGFβ. This demonstrates that Mitf function is closely linked to the relationship between transcription signature and *in vitro* phenotype, confirming it as a useful *in vivo* marker for identifying different signature cells.

Proliferative Cells Form Fast Growing Tumors Sooner Than Invasive Cells.

To test the relationship of cell line signature assignments with *in vivo* behaviour we performed subcutaneous injection of cell lines into the flanks of immunocompromised mice and recorded tumor growth characteristics. We found that proliferative melanoma lines consistently formed tumors about two weeks after being injected into the flanks of athymic nude mice, while invasive lines took considerably longer to begin tumorigenesis (Figure 2A). The length of time taken for growth initiation, measured as the time at which tumor volume exceeds 100 mm^3, was 14 ± 3 days for proliferative signature cells, and 59 ± 11 days for invasive signature cells ($p < 0.001$). These data provide *in vivo* evidence for the significance of a proliferative signature in melanoma cells as predicted by *in vitro* experiments. The

proliferative signature seeded tumors all initiated growth at nearly the same time point. Contrasting this, initiation times for the invasive signature seeded tumors was spread over a wider period. This suggests to us that proliferative signature seeded initiation is less dependent on microenvironment variation than invasive signature seeded initiation.

Tumors Derived From Proliferative Or Invasive Lines Are Indistinguishable.

Because both transcription signature melanoma cell types yielded tumors we were interested in examining these for signature-specific differences. Upon excision the tumors were stained for Mitf and Ki67 antigen expression to look at the distributions of these markers. Tumors derived from invasive signature cell lines, which did not stain for Mitf, revealed melanoma cells with nuclei which were Mitf-positive and melanoma cells with nuclei which were Mitf-negative (Figures 5A-E). Tumors derived from proliferative signature cell lines, which stained for Mitf, showed the same patterning of stained and unstained melanoma cell nuclei (Figures 5F-J). Additionally, in all tumors examined we found that Mitf-stained nuclei tended to concentrate within the peripheral margins of the tumors. We found that Ki67 antigen staining patterns were similarly indistinguishable in tumors derived from proliferative or invasive signature lines. Conversely, it was apparent that tumor regions showing Mitf-positive nuclei were also enriched for Ki67-positive nuclei. These findings showed that tumors seeded with invasive or proliferative signature cell lines were not distinguishable and that homogeneous *in vitro* staining patterns yielded strikingly heterogeneous patterns *in vivo*, showing that signature patterns of melanoma cells change bi-directionally *in vivo*.

DISCUSSION

A feature of current models for gene expression involvement in melanoma progression is their explicitly one-way nature. It is typical to present a graph in which gene expression changes proceed concomitantly with stage progression, where a gene either increases or decreases expression as the disease evolves through clinically recognized stages to metastasis (Miller and Mihm, 2006). However, models of this design do not account for the broad molecular heterogeneity which is apparent in melanoma. Immunohistochemical studies do not support the idea that the expression of certain genes is switched irrevocably on or off during the course of the disease, starting from one state at transformation and ending at another once metastasis has been achieved. Instead it is clear that, in many melanoma cells within a given lesion, genes thought to be downregulated in late stages are shown to persist and others thought to be upregulated are absent. One possible answer is that many of the genes associated with metastatic potential may not undergo one-way modification of regulation and instead retain the potential to reverse changes in expression.

Investigations into the gene expression signatures of melanoma cell lines taken from late stage tumors show that a given cell line is highly likely to express one of two major transcription programs. It was also determined that the genes whose expression patterns respectively delineated the two signatures were likely involved in melanoma metastatic potential (Bittner et al., 2000; Hoek et al., 2006). One of these signatures (identified by us as *proliferative*) has *MITF* and other melanocytic genes (e.g. *TYR*, *DCT*, *MLANA*) upregulated along with a number of additional neural crest-related factors (e.g. *SOX10*, *TFAP1A*, *EDNRB*), and this signature is associated with high rates of proliferation, low motility and sensitivity to growth inhibition by TGFβ. A second signature (identified by us as *invasive*) downregulates these genes and instead upregulates others whose secreted products (e.g. *INHBA*, *COL5A1*, *SERPINE1*) are known to be involved in modifying the extracellular environment, and this

signature is associated with lower rates of proliferation, high motility and resistance to growth inhibition by TGFβ. Having identified these genes we found that many of the proliferative signature were common responders to Wnt signaling, and those of the invasive signature were TGFβ signal-driven, and we proposed that these signaling pathways are responsible for the different transcription signatures observed (Hoek et al., 2006). Among genes comprising the invasive signature are several (e.g. *WNT5A*, *DKK1* and *CTGF*) known to negatively regulate Wnt signaling (Ishitani et al., 2003; Mercurio et al., 2004; Zorn, 2001), suggesting that activation of TGFβ signaling may precipitate deactivation of Wnt signaling. Similar cross-talk opposition between TGFβ and Wnt signaling has already been noted in gastrointestinal cancer (Mishra et al., 2005). This link between the signatures indicated to us that they may be reversible given appropriate signals, and further suggested that proliferation and invasion are program states which melanoma cells activate according to microenvironmental cues.

The results of our *in vitro* proliferation and motility analyses were consistent with signature assignments inferred from earlier DNA microarray experiments (Hoek et al., 2006). In order to immunohistochemically differentiate signatures *in vivo* we used nuclear Mitf as our marker for the proliferative signature and nuclear Ki67 antigen as a general indicator for proliferation activity. We found *in vitro* that while both invasive and proliferative signature melanoma cells expressed Ki67 antigen, significantly more proliferative signature cells expressed it, correlating with the relative growth differences observed between proliferative and invasive cells *in vitro*. The Ki67 antigen is preferentially expressed during late G1, S, G2 and M phases of the cell cycle and is not detected in G0 (Braun et al., 1988; Bruno and Darzynkiewicz, 1992), that Ki67 antigen may be an absolute requirement for cell proliferation was indicated by siRNA knockdown experiments in myeloma cells (Schluter et al., 1993). The role of Mitf in melanoma proliferation is less clear. One report notes that Mitf expression in melanoma is suppressed by BRAF activation and increasing Mitf expression in this context

is anti-proliferative (Wellbrock and Marais, 2005). However, increasing Mitf expression in melanoma has also been shown to be a proliferative factor and is involved in Cdk2 production (Du and Fisher, 2002; Widlund et al., 2002). Recent studies have shown that Mitf is also an indirect negative regulator of the $p27^{Kip1}$ Cdk inhibitor, which would otherwise act to suppress Cdk2 activation and block G1/S transition (Carreira et al., 2006). Our data also supports a proliferative role for Mitf *in vitro* as we find that Mitf-positive lines proliferate faster (Figure 1) and express Ki67 antigen with greater frequency than Mitf-negative lines (Figure 2).

We performed siRNA knockdown of Mitf in a proliferative signature cell line and show that this confers a phenotype observed in invasive signature line melanomas. Proliferative signature cells are susceptible to TGFβ-mediated inhibition of proliferation, while invasive signature cells are not. Several other studies also showed that aggressively invasive melanoma cells are resistant to TGFβ (Heredia et al., 1996; Krasagakis et al., 1994; Roberts et al., 1985). Our DNA microarray data suggested that Mitf gene expression is central to the proliferative signature and may therefore have a role in mediating the growth-inhibitory response to TGFβ. After knockdown of Mitf expression in a proliferative signature line the cells were less susceptible to TGFβ-mediated inhibition of proliferation (Figure 2). Experiments by others have shown that invasion through matrigel is increased in Mitf-expressing melanoma cells treated with siRNA targeting Mitf (Carreira et al., 2006). Together these combined findings indicate that regulation of Mitf expression is critical to signature membership and supports our contention that *in vivo* changes in nuclear Mitf staining indicate proliferative/invasive signature switching.

Our xenograft experiments showed that the proliferative and invasive gene expression signatures also correlated appropriately with tumor growth patterns. Proliferative signature lines initiated tumors about two weeks after injection while invasive signature lines lay

dormant for an average of eight weeks before tumor growth began (Figure 2). While these and the *in vitro* experiments further support the different signature assignments to different melanoma cell lines, immunohistochemical examination of the tumors could not readily distinguish between them, rather they showed evidence for signature switching. Comparison of *in vitro* Mitf staining patterns with *in vivo* Mitf staining patterns shows that resultant tumors deriving from *either* proliferative signature or invasive signature lines reveal the presence of both Mitf-positive and Mitf-negative melanoma cells (Figure 3). Concurrently, Ki67 antigen staining, while found throughout the tumors, was significantly more frequent in regions positive for Mitf staining than in regions absent of Mitf. Furthermore, the distribution of Mitf staining and increased frequency of Ki67 antigen positivity shows a distinctly peripheral pattern. This suggests that melanoma cells proximal to the interface between host tissues and the tumor are actively undergoing increased rates of proliferation.

The long lag-time for tumor initiation observed with invasive signature cells and the presence of Mitf-positive nuclei in resulting tumors indicate that tumor growth was probably preceded by a switch in some cells to the proliferative signature type. Similar microenvironment-driven signature switching has been shown previously in other experiments. Recent studies investigating the effect of embryonic environments on melanoma cells has shown how these environments can affect the aggressive phenotype. Kulesa and co-workers, using the aggressive C8161 melanoma line, showed that transplantation of C8161 cells into chick embryonic tissues stimulated re-expression of melanocytic markers similar to poorly aggressive cells (Kulesa et al., 2006). Complimentary *in vitro* studies in which poorly aggressive cells, grown on 3D matrices preconditioned by aggressive lines, showed upregulation of extracellular matrix modifying genes and increased invasive ability (Seftor et al., 2006). Additional experiments in zebrafish revealed that in embryonic environments inhibition of the morphogen Nodal switched melanoma cells to a less aggressive phenotype,

suggesting that Nodal signaling (which acts through TGFβ family receptors) was important to maintaining aggressive phenotypes in melanoma cells (Topczewska et al., 2006). Signature switching of cells in response to the microenvironment would explain why our xenograft tumors deriving from different signature lines were immunohistochemically indistinguishable. That rapidly proliferating cells are found closer to the periphery of growing tumors also supports a role for the microenvironmental determination of activity.

These data are critical pieces of the melanoma progression puzzle because they suggest not only that invasion and proliferation are divisible aspects of metastatic potential, but that these different transcriptional states are interchangeable programs between which melanoma cells oscillate during progression in response to changing microenvironmental cues (Figure 5). What these microenvironmental cues precisely are remains unknown, but there is growing evidence that hypoxia may be one (Holmquist et al., 2006) and inflammation another (de Visser et al., 2006). The relevance of this model to clinical aspects of melanoma is that it may explain why metastatic melanoma is so resistant to chemo- and immunotherapeutic strategies. The heterogeneity inherent in tumors containing melanoma cells whose transcriptional patterns and biological activities are dependent on the microenvironment suggests that while proliferating cells are susceptible to chemotherapy there are a population of cells which, though not proliferating, have the capacity to switch to a proliferative program and successfully drive tumor progression once therapy has ceased. By the same token, targeted therapies which frequently aim for melanocytic antigen-expressing cells would not recognize cells whose expression programs are dedifferentiated from the melanocytic phenotype, cells who will serve to repopulate that phenotype at a later time.

Experimental procedures

Melanoma tissues and lines

Melanoma cell cultures were established from surplus material from cutaneous melanoma metastases removed by surgery after having obtained written informed consent of the patient. Clinical diagnosis was confirmed by histology and immunohistochemistry. Melanoma cells were released from tissue sections and grown as previously described (Geertsen et al., 1998). Cell lines were chosen according to their transcription pattern signatures as previously described (Hoek et al., 2006). Two proliferative signature (M980513, M000907) and two invasive signature (M991121, M010308) melanoma lines were used. Paraffin-embedded examples of cutaneous metastases of melanoma were also selected for comparative analysis.

In vitro motility and proliferation assays

For the motility assays 2 x 10^4 melanoma cells were seeded on 8μM transwell microporous filters (Becton Dickinson, Franklin Lakes, NJ) in 200 μl RPMI. As a chemoattractant RPMI containing 10% FCS was added to the lower chamber. After 18 hours of incubation cells on the upper side of the filter were removed with cotton swab. The membrane was then stained using a standard hematoxylin and eosin protocol and the cells were counted under a light microscope. For the proliferation assay melanoma cells were seeded to a density of 5 x 10^4 in each well of a six-well plate. After 24, 72 and 96 hours cells were counted in a Neubauer chamber to estimate cell-doubling times.

Recombinant adenovirus vector and siRNA

Recombinant first-generation, E1/E3-deleted Ad5-based vectors Ad-H1-siMitf and Ad-H1-siControl were generated as described previously(Hemmi et al., 1998). Briefly, homologous recombination was performed in human embryonic retinoblast line 911 cells between a transfer plasmid pAd-H1-siMitf encoding the Mitf-specific siRNA sequence under the control

of the H1 promoter and a genomic *Cla*I DNA fragment isolated from AdMLP-*lacZ*. To construct pAd-H1-siMitf, the CMV promoter of pAd-CMVΔlacZ-lnk1 was replaced with the H1 promoter (Hasuwa et al., 2002). The H1 promoter fragment was PCR-amplified from genomic DNA of human 293T cells and cloned into *Sfi*I/*Bam*HI-restricted pAd-CMVΔlacZ-Ink1. Following this, oligonucleotides for the silencing cassette(Saydam et al., 2005) containing a 19-nucleotide siRNA sequence targeting Mitf (Busca et al., 2005) were cloned into *Nhe*I/*Sal*I-restricted pAd-H1ΔlacZ-lnk1 (Ad-H1-siMitf). For a mock control (Ad-H1-siControl), the siRNA sequence of Mitf was scrambled and blasted to ensure no human sequence is targeted. Recombinant adenoviruses were plaque-purified, amplified, and CsCl purified. Viral titers were determined by plaque assay, using 911 cells, and were 1.8×10^{10} PFU/ml for Ad-H1-siMitf and 1.3×10^{10} PFU/ml for Ad-H1-siControl.

Transfection and TGFβ challenge assay

Melanoma cells were seeded to a density of 4×10^4 cells in a 24-well plate one day before infection. The next day medium was changed to RPMI containing 2% FCS and cells were either infected with virus particles carrying the pAd-H1-siMitf or pAd-H1-siControl. For assessment of susceptibility to growth inhibition by TGFβ, cells were challenged with 5 ng/ml recombinant TGFβ (Biosource, Camarillo, CA) 24 hours after virus transduction. After a further 56 hours cell growth was estimated using a standard MTT assay.

Western blot analyses

Cells were solubilized in lysis buffer containing 20 mM Tris-HCl pH 7.5, 1% TritonX-100, 150 mM NaCl, 10% glycerol and Complete mini protease inhibitor (Roche Diagnostics GmbH, Mannheim, Germany). Proteins were separated on a NuPAGE 10% Bis-Tris gel (Invitrogen, Carlsbad, CA) under denaturing and reducing conditions followed by transfer onto a Nitrocellulose membrane (Invitrogen). Mitf protein was detected with a mouse anti-

Mitf MAb (LabVision, CA, USA) diluted 1:100 in 3% BSA at 4^{o}C overnight. Secondary rabbit-anti-mouse antibodies (Abcam, Cambridge, UK) conjugated with peroxidase was used at a dilution of 1:10000. Detection by chemiluminescence used an enhanced chemiluminescence system (GE Healthcare, Buckinghamshire, UK).

Xenografts

For each melanoma line a total of 3 x 10^6 cells were injected into both flanks of eight-week old female athymic nude mice. Mice were kept in individually ventilated cages for a maximum of 75 days post injection. Size and mass of tumors was recorded once every 3-7 days until linear growth was detected, after which measurements were taken every 1-2 days. If at least one xenograft tumor reached 1 cm^3 the mouse was sacrificed and tumors removed. If the condition of the mouse deteriorated (e.g. listlessness, loss of weight) the mouse was sacrificed and tumors removed. All remaining mice were sacrificed on the 75th day and tumors removed. Effective tumor initiation time was calculated on the day tumor volume reached 100 mm^3. Tumors not reaching 100 mm^3 within 75 days were not considered.

Immunohistochemistry

Cell lines were prepared for immunohistochemistry as follows. Briefly, cells were cultured, washed in PBS (Biochrom, Berlin, Germany) and then put into suspension by incubating in 2 mL trypsin/EDTA solution (Biochrom) at 37° C. Trypsin was inactivated by adding 18 mL of FCS-containing growth medium. Cell suspensions were centrifuged for 5 min at 2000 rpm. After removing the supernatant, four drops of plasma were added to the pellet and the solution was mixed. One drop of thrombin was added and after five minutes the coagulated material was encapsulated for fixation in 4% formalin and embedded in paraffin. Excised xenograft samples were fixed in 4% formalin and embedded in paraffin. Slides were cut from paraffin blocks and immunohistochemically stained using the alkaline phosphatase-anti-alkaline

phosphatase technique and counter-stained using hematoxylin. Antibodies used were directed against Mitf or Ki-67 (DakoCytomation, Glostrup, Denmark). Counting of stained and unstained nuclei was done on a PC using the free UTHSCSA ImageTool program (developed at the University of Texas Health Science Center at San Antonio, Texas and available from the Internet by anonymous FTP from ftp://maxrad6.uthscsa.edu).

Statistical analysis

For all quantitative sample comparisons Student's two-sample heteroscedastic t-test was used to calculate a t-statistic for comparison against a significance cutoff of $p = 0.05$.

ACKNOWLEDGEMENTS

We thank Christa Dudli for technical assistance with the immunohistochemical analyses. This work was supported by funds obtained from the Swiss National Foundation (grant no. 310040-103671) and the Gottfried and Julia Bangerter Rhyner Stiftung.

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FIGURE LEGENDS

Figure 1. *In vitro* correlations with gene expression signatures. M980513 and M000907 proliferative signature (Pro) melanoma lines, as well as M991121 and M010308 invasive signature (Inv) melanoma lines, were chosen for this study. (A) A gene expression heatmap, generated by clustering samples based on the normalized expression of 105 metastatic potential genes, shows the subtype-specific signatures for Pro and Inv signature melanoma lines. *In vitro* growth (B) and motility (C) experiments correlate appropriately with proliferative and invasive signature assignments. Error bars indicate standard deviation.

Figure 2. siRNA knockdown of Mitf protects against TGFβ-mediated growth inhibition. (A) siRNA-mediated knockdown of Mitf in a proliferative signature melanoma cell line was confirmed by Western blot analysis. (B) TGFβ-susceptibility experiments show that Mitf knockdown promotes significant ($p < 0.03$) resistance to TGFβ-mediated growth inhibition in a proliferative signature melanoma cell line.

Figure 3. Immunohistochemical marker correlations with gene expression signatures. Immunohistochemical analysis of paraffin-embedded cell lines shows that proliferative and invasive signature lines have differential staining for Mitf (93% and 0%, respectively) and Ki67 antigen (92% and 45%, respectively).

Figure 4. Xenograft tumor growth. Human melanoma cell lines were injected into both flanks of immunocompromised nude mice. Proliferation of melanoma cells led to tumor growth which was monitored daily. Proliferative melanoma cells (Pro) formed tumors rapidly, while invasive melanoma cells (Inv) took weeks longer to initiate tumor growth.

Figure 5. Immunohistochemistry of melanoma xenograft tumors. Cell lines were injected into the flanks of immunocompromised nude mice and allowed to grow tumors for a maximum of 75 days. After a tumor had formed it was removed and subjected to immunohistochemical analysis. (A) Tumor resulting from an invasive signature melanoma (M010308), with two separate fields (1 and 2) examined in more detail. (B and C) Mitf and Ki67 stains of field A1. (D and E) Mitf and Ki67 stains of field A2. (F) Tumor resulting from a proliferative signature melanoma (M980513) line. (G and H) Mitf and Ki67 stains of field F1. (I and J) Mitf and Ki67 stains of field F2.

Figure 6. An integrated model for gene regulation of melanoma metastatic potential and progression. Early phase melanoma cells expressing the "proliferative signature" gene set proliferate to form the primary lesion. Following this an unknown signal switch, likely brought about by altered microenvironmental conditions (e.g. hypoxia), gives rise to cells with a significantly different "invasive signature" gene set. Invasive signature cells escape and, upon reaching a suitable distal site, revert to the proliferative state and nucleate a new metastasis where the cycle is repeated. Each switch in phenotype (state change) is accompanied by an exchange in expressed gene sets from proliferative to invasive and *vice versa*.